Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Testing of Erysipelas Bacterins in Mice

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Contact Person: Charles Egemo, (515) 663-7407

Approvals:

/s/ Linda Schlater Date: 20 Dec 99
Linda K. Schlater, Head
Biologics Bacteriology Section

/s/ Ann L. Wiegers Date: 22 Dec 99
Ann L. Wiegers, Quality Assurance Manager

/s/ Randall L. Levings Date: 12/21/99
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing *Erysipelothrix rhusiopathiae*, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.119. Mice are vaccinated and then challenged with a standard dose of virulent *E. rhusiopathiae* 14-21 days after vaccination.

1.2 Keywords

Erysipelothrix rhusiopathiae, erysipelas, mouse, potency, vaccination-challenge, bacterin, 9 CFR 113.119

2. Materials

2.1 Equipment/instrumentation

2.1.1 Spectrophotometer, Spectronic 70™ (Bausch and Lomb, Rochester, New York) or equivalent

2.1.2 Bunsen burner

2.1.3 Incubator, 36°-38°C

2.1.4 Automatic pipetting device, or pipette bulb

2.1.5 Crimper for aluminum caps on serum vials

2.2 Reagents/supplies

2.2.1 *E. rhusiopathiae* strain E1-6 challenge culture, IRP ERC, current lot. This culture is available from the Center for Veterinary Biologics-Laboratory (CVB-L), United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Ames, IA.

2.2.2 Test bacterin(s) containing *E. rhusiopathiae*

2.2.3 APHIS-approved *E. rhusiopathiae* reference bacterin, IRP ERB, current lot, is available from the CVB-L, USDA-APHIS, Ames, IA.

2.2.4 Syringes, 1 ml and 3 ml

2.2.5 Needles, 26 ga, 3/8 in and 20 ga, 1 in

2.2.6 Glass serum bottle, 20 to 100 ml

2.2.7 Rubber stopper, 13 x 20 mm, and aluminum cap for serum bottle

2.2.8 Glass screw-top tubes, 13 x 100 mm, with caps

2.2.9 Pipettes, 5 ml and 25 ml

2.2.10 Erysipelas challenge culture medium

2.2.11 Bovine blood agar plates

2.2.12 Peptone buffer

2.2.13 Saline, 0.85%

2.2.14 Water, distilled or deionized, or water of equivalent purity

2.3 Animals

2.3.1 Mice, 16-22 g. **Note**: **Although the 9 CFR does not specify a specific mouse type or sex, the CVB-L uses CF-1 female mice.**

2.3.2 Eighty mice are required for each serial to be tested (20 mice/dilution x 4 dilutions/serial). Eighty additional mice are required for the reference bacterin. **Note: Although 9 CFR regulations require only 3 dilutions/serial, the CVB-L tests an additional**

dilution to increase the probability of bracketing the PD_{50} dilution. Thirty mice are required to determine the LD_{50} of the challenge inoculum. All mice must be from the same source colony and of similar weight and/or age.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel must have working knowledge of the use of general laboratory chemicals, equipment, and glassware and have specific training and experience in sterile technique, the handling of live bacterial cultures, and the handling of mice.

3.2 Selection and handling of test mice

3.2.1 Mice of either sex may be used, but females are recommended.

3.2.2 All mice must be housed and fed in a similar manner.

3.2.3 Identify each cage of mice by treatment group.

3.2.4 If any mice die after vaccination but prior to challenge with live *E. rhusiopathiae*, necropsy these mice to determine cause of death if the cause of death is not outwardly apparent. If the cause of death is unrelated to vaccination, file the necropsy report with the test records and no additional action is needed. If death is attributable to the test bacterin, report the death immediately to the Center for Veterinary Biologics-Inspection and Compliance (CVB-IC). **Note: CVB-IC may request follow-up safety testing of the bacterin if mice die at this stage.**

3.2.5 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the mice and to sanitize contaminated rooms.

3.3 Preparation of supplies/equipment

3.3.1 Sterilize all glassware before use.

3.3.2 Use only sterile supplies (pipettes, syringes, needles, rubber stoppers, diluents, etc.).

3.3.3 All equipment must be operated according to manufacturers' instructions and maintained and calibrated, as applicable, according to current CVB-L Standard Operating Procedures (SOPs).

3.4 Preparation of reagents

3.4.1 *E. rhusiopathiae* bacterin. Reference bacterin IRP ERB, current lot. Make threefold dilutions in saline according to directions on the accompanying reagent data sheet immediately prior to use. Place each of the dilutions in separate sterile serum bottles. Typically, the CVB-L tests the bacterins at 1:10, 1:30, 1:90, and 1:270 dilutions. It is permissible to make threefold dilutions other than those described as long as the reference and test bacterins are tested at the same dilutions.

3.4.2 Test bacterin(s) containing *E. rhusiopathiae*. For each test bacterin, make threefold dilutions in the appropriate diluent (see **Section 4.1.3)** immediately prior to use. Use dilutions identical to the dilutions used for the Reference bacterin (see **Section 3.4.1).** Place each of the dilutions in separate sterile serum bottles.

3.4.3 *E. rhusiopathiae* challenge culture. The challenge culture, IRP ERC, is lyophilized in 0.2-ml amounts. Store vials of lyophilized culture at ≤7°C.

3.4.4 Saline, 0.85% (National Veterinary Services Laboratories [NVSL] media 30201)

Sodium chloride	8.5 g
Water	q.s. to 1000 ml

Autoclave 20 min at 121°C. Store at room temperature for no longer than 1 yr.

3.4.5 Peptone buffer (NVSL media 10522)

Peptone	10 g
Sodium phosphate, dibasic	12.01 g
Potassium phosphate, monobasic	2.09 g
Water	q.s. to 1000 ml

Adjust pH to 7.3-7.5. Autoclave 20 min at 121°C. Cool before using. Store at room temperature no more than 6 mo.

3.4.6 Bovine blood agar (NVSL media 10006)

Blood agar base powder	40 g
Water	q.s. to 950 ml

Autoclave 20 min at 121°C. Cool to approximately 47°C. Add 50 ml defibrinated bovine blood. Pour into sterile petri dishes. Cool to room temperature. Store at 2°-7°C for no more than 6 mo.

3.4.7 Erysipelas challenge culture medium (NVSL media 10133)

Horse meat (no fat)	454 g
Horse liver	18 g
Water	1000 ml

Grind tissue and dispense in hot water in a cooker. Heat to boiling and simmer for approximately 1 hr. Allow to settle at least 2 hr. Skim off fat and discard meat. Strain through cheese cloth. Filter through No. 2 Whatman filter paper.

Combine 1000 ml of filtrate with:

Sodium phosphate, monobasic	11 g
Potassium phosphate, monobasic	1 g
Bile (fresh frozen fluid)	10 ml
OR Oxgall® (1 g in 10 ml H_2O)	
Peptone	20 g
Gelatin, granulated	5 g

Heat to just below boiling to dissolve the gelatin. Cool to approximately 56°C. Adjust pH to 7.8.

Add:

Dextrose	5 g
Horse serum (not heat inactivated)	100 ml

Filter while still hot through a sterile, disposable .2-μm mini capsule filter. Adjust final pH to 7.6-7.8 with filtered acid or base. Store at 2°-7°C for no longer than 6 mo.

4. Performance of the test

4.1 Vaccination of test animals

4.1.1 Check the label on each product to confirm identity and dose volume.

4.1.2 Test each test bacterin and the reference bacterin at a minimum of 3 threefold dilutions.

4.1.3 Thoroughly mix product by inverting end-to-end. Make the appropriate threefold dilutions of the reference bacterin in saline. Make identical threefold dilutions of the test bacterin(s) in saline or the diluent approved in the specific outline of production for that product. (Some oil-adjuvanted products require oil-based diluents.) Place each dilution in a separate sterile serum bottle. Prepare dilutions immediately prior to use; do not store in diluted form.

4.1.4 Weigh 5 randomly selected mice immediately prior to vaccination to assure that the average body weight of the mice is between 16 and 22 g. Record weights.

4.1.5 Vaccinate separate groups of 20 mice with each of the test bacterin dilutions and reference bacterin dilutions. For reference bacterin groups, inject each mouse with 0.2 ml subcutaneously. Inject test bacterins subcutaneously at a dose volume that corresponds to 1/10 of the smallest dose recommended on the product label. This volume must not be <0.1 ml.

4.1.6 Retain 30 nonvaccinated mice to determine LD_{50} of the challenge.

4.2 Preparation of challenge

4.2.1 Reconstitute a vial of challenge in 1.5 ml peptone buffer.

4.2.2 Inoculate 10 ml of erysipelas challenge culture medium with the entire contents of a vial of reconstituted culture.

4.2.3 Incubate the inoculated broth at 36°-38°C for 18-20 hr.

4.2.4 Perform a Gram stain, according to current version of BBSOP0004, on the overnight cultures. If the Gram stain shows a pure culture of Gram-positive rods, continue with the challenge procedure. If the culture is not pure, begin the preparation of the challenge again. (**Section 4.2.1**)

4.2.5 Dilute overnight culture as necessary in sterile erysipelas challenge culture broth to 70% ± 2% T at 600 nm, using a spectrophotometer.

Note: Use sterile erysipelas challenge culture medium as a blank for the spectrophotometer.

4.2.6 Prepare a 10^{-5} dilution of the standardized culture in sterile erysipelas challenge medium broth. **Note: This is the inoculum used to challenge the mice and is hereafter referred to as "challenge inoculum."** Place the challenge inoculum in a serum vial and seal it with a rubber stopper and aluminum ring. Save an aliquot(s) of this inoculum in a separate vial(s); retain vial(s) as a sample for postchallenge plate counts.

4.2.7 Make 4 additional tenfold dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of the challenge inoculum to determine LD_{50} of the challenge. Place each dilution in a separate labeled serum vial and seal the vials.

4.2.8 Place all vials of challenge inoculum on ice to transport to the animal room. Keep the challenge inoculum on ice through the challenge procedure and until the culture is added to the blood agar plates for the postinoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge all vaccinated mice 14-21 days after the vaccination.

4.3.2 Challenge the nonvaccinated LD_{50} control mice at the same time as the vaccinated mice.

4.3.3 Inoculate each vaccinated mouse with 0.2 ml of challenge inoculum subcutaneously, using a 1-ml syringe with a 26-ga x 3/8-in needle.

4.3.4 Inoculate 1 group of 10 nonvaccinated control mice subcutaneously with 0.2 ml of the challenge inoculum. Inoculate 4 groups of 5 mice each with the 4 dilutions of the challenge inoculum.

4.4 Postinoculation plate count

4.4.1 After the mice are challenged, perform a colony count on blood agar plates according to the current version of BBSOP0019, using the vials retained for this purpose.

1. Use sterile erysipelas challenge culture broth as the diluent for the plate count, and plate the challenge inoculum dilutions on bovine blood agar. Incubate the plates aerobically at 36°-38°C for 48-72 hr.

2. Calculate the colony-forming units (CFU) per challenge dose according to the following formula:

Note: Average count in 0.1 ml culture x 2 x dilution factor (see table below)=CFU/0.2-ml dose of challenge culture.

If plates used for avg. count were inoculated with:	Dilution factor
10^{-1} dilution of challenge inoculum	10
10^{-2} dilution of challenge inoculum	100
10^{-3} dilution of challenge inoculum	1000
10^{-4} dilution of challenge inoculum	10000

4.5 Observation of mice after challenge

4.5.1 Observe the mice daily for 10 days after challenge. Record deaths.

4.5.2 If deaths occurring after challenge are suspected to be due to causes other than erysipelas, necropsy the mice to determine the cause of death. If cause of death is unrelated to vaccination and/or challenge, do not include the deaths in the total deaths for the test.

5. Interpretation of the test results

5.1 Interpret the test as prescribed in 9 CFR, Part 113.119.

5.1.1 Calculate the LD_{50}/dose (theoretical dilution at which the challenge would be lethal to 50% of the control mice) of the challenge inoculum using the Reed-Muensch method of estimation. For a test to be valid, the challenge inoculum must contain at least 100 LD_{50}/0.2 cc dose.

5.1.2 Calculate the PD_{50} of the reference bacterin and each test bacterin (theoretical dose/dilution at which the bacterin would protect 50% of the mice) using the Reed-Muensch method of estimation.

5.1.2.1 If the PD_{50} of the reference cannot be calculated because the lowest dilution tested protects <50% of the mice, the serial may be retested, ***provided that*** the following conditions are met:

1. If the serial is not retested, it is unsatisfactory.

2. If the protection provided by the lowest dilution of the reference exceeds that provided by the lowest dilution of the test serial by at least 6 mice, the test serial is unsatisfactory without additional testing.

3. If the total number of mice protected by the reference (sum of survivors in all dilution groups) exceeds the total number protected by the test serial by 8 mice or more, the test serial is unsatisfactory without additional testing.

5.1.2.2 If the PD_{50} of the test serial in a valid test cannot be calculated because the highest dilution protected more than 50% of the mice, the serial is satisfactory without further testing.

5.1.3 Divide the PD_{50} of each test serial by the PD_{50} of the reference to calculate the relative potency (RP) for each serial.

5.1.4 If the RP of the test serial(s) is <0.60, the test serial is unsatisfactory.

5.1.4.1 A test serial with an RP <0.60 may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. Calculate the results of the retests in the following manner:

1. Average the RP values of the retests.

2. If the average RP of the retests is <0.60, the serial is unsatisfactory.

3. If the average RP of the retests is ≥0.60 **AND** the RP obtained in the original test is 1/3 or less of the average RP of the retests, the test bacterin is satisfactory. Consider the initial test to be the result of test system error.

4. If the average of the retests is ≥0.60 **BUT** the RP of the original test is greater than 1/3 of the average RP of the retests, calculate a new average RP using the RP values obtained in all tests (original plus

retests). If the new average RP is ≥0.60, the test bacterin is satisfactory. If the new average RP is <0.60, the test bacterin is unsatisfactory.

5.1.5 Record the plate count (CFU/dose) of the challenge on the test result form for informational purposes to track trends and to troubleshoot problem tests. The 9 CFR does not specify a minimum or maximum CFU/dose for this test.

6. Report of test results

Report the results of the test(s) as described in the current version of BBSOP0020.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.119, U.S. Government Printing Office, Washington, DC, 1999.

7.2 Reed LJ, Muench H, 1938. A simple method of estimating 50% endpoints. *Am J Hygiene*, 27:493-497.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.